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Genetically Encodable Fluorescent and Bioluminescent Biosensors Light up Signaling Networks

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Abstract

Cell signaling networks are intricately regulated in time and space to determine the responses and fates of cells to different cues. Genetically encodable fluorescent and bioluminescent biosensors enable the direct visualization of these spatiotemporal signaling dynamics within the native biological context, and have therefore become powerful molecular tools whose unique benefits are being used to address challenging biological questions. Here, we first review the basis of biosensor design and remark on recent technologies that are accelerating biosensor development. We then discuss a few of the latest advances in the development and application of genetically encodable fluorescent and bioluminescent biosensors that have led to scientific or technological breakthroughs.

Keywords

Spatiotemporal regulation; compartmentalization; live-cell imaging; super-resolution; bioluminescent; in vivo imaging

Biosensors illuminate signaling dynamics

Cells respond to a variety of internal and external cues through a number of signaling pathways that engage in complex, coordinated, and often dynamic networks [1]. These signaling networks are fundamental to determining proper cellular responses and functions, as well as for processing signal inputs at the systems level to maintain the well-being of the organism. In recent years, growing evidence has suggested that a large number of cell signaling events, including the activation of kinases and phosphatases, recruitment of **second messengers** (see Glossary), and modulation of protein-protein interactions, depend on discrete spatial distribution and precise temporal regulation to produce diverse physiological outcomes [2, 3]. The spatiotemporal aspects of signaling, however, are challenging to track properly and visualize dynamically within their native cellular context.

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To meet these demands, a wide array of **genetically encodable fluorescent and bioluminescent biosensors** has been developed and tailored to visualize specific signaling events in a rapid, sensitive and non-invasive way [4]. The state-of-the-art biosensor toolbox has been employed, in conjunction with advanced live-cell imaging techniques, to monitor and quantify signaling dynamics with spatiotemporal precision within the endogenous cellular environment (Table 1). As powerful molecular tools for elucidating the molecular and cellular basis of biological processes, biosensors offer unique advantages, including genetic targetability, high spatial and temporal resolution, and the capability of providing quantitative live-cell information.

Over the past 20 years, advances in biosensor development have constantly pushed the boundaries of achieving higher spatiotemporal resolution for imaging biological processes, such as signaling pathways in live cells, tissues and intact organisms. Here, we review the latest progress in the development and application of biosensors to address challenging biological questions, focusing on superresolution imaging of kinase signaling, ultrafast neurotransmitter signaling dynamics, and in vivo bioluminescence imaging. We highlight a few examples and discuss how future efforts can fill technological gaps and lead to new discoveries.

Recent Advances in Biosensor Development

The design of the sensing and reporting units (Box 1) often holds the key to successfully developing biosensors with high sensitivity and fidelity. Implementation of new designs and new reporting units obtained through protein engineering has recently helped to greatly enhance biosensor performance, such as by increasing dynamic range, signal-to-noise ratio (SNR), and the capability for multiplexing or in vivo imaging. For example, Mehta and coworkers recently reported a generalizable backbone for a suite of single-fluorophore KARs in various colors for sensitive and multiplexed tracking of signaling activities [5]. In the case of the excitation ratiometric A kinase activity reporter, ExRai-AKAR (Figure 1A), phosphorylation by PKA induces a conformational change of the reporter, which modulates the fluorescence excitation spectrum of **circular permuted GFP** (**cpGFP**) and leads to a specific shift in the excitation peak of cpGFP from ~400 nm to ~480 nm. ExRai-AKAR, which utilizes the ratio of the fluorescence intensities at these two excitation wavelengths as a readout for kinase activity, was found to exhibit 3- and 2-fold increases in dynamic range and SNR compared to the best performing FRET-based PKA reporter, AKAR4. Using these probes with different colors and subcellular targeting, they were also able to track six-fold multiplexed imaging of distinct signaling activities simultaneously, such as PKA/cAMP/ERK/Ca^{2+}, at different subcellular localizations in single living cells [5].

Parallel efforts in fluorescent protein (FP) engineering have improved brightness [6] and even yielded entirely new reporting units, such as **infrared fluorescent proteins** (**IFPs**) [7–9]. Owing to their near-infrared (NIR) excitation and emission wavelengths (~650–900 nm), IFPs open new avenues for deep-tissue and in vivo fluorescence imaging [8, 10, 11] by minimizing autofluorescence, absorption and scattering of the excitation and emission light by endogenous cellular components [12]. Extending the color palette of IFPs further enables the development of NIR-FRET biosensors based on the aforementioned modular design. In

recent work by Shcherbakova and colleagues, the first fully NIR-FRET pair, miRFP670 and miRFP720, was utilized to generate NIR-FRET biosensors for Rac1 GTPase (Figure 1B), as well as the kinases PKA and JNK, that are spectrally compatible with common CFP-YFP FRET biosensors [13]. This allowed the authors to perform multiplexed FRET imaging and directly visualize the antagonism between Rac1 and RhoA in the same cell during cell-edge movement [13].

On the other hand, the specificity and fidelity of biosensors largely rely on the sensing unit, typically a protein or peptide that undergoes an intrinsic or engineered conformational change in response to biological events. For example, to design a kinase activity reporter, it is critical to identify a substrate peptide specific to a kinase-of-interest as part of a sensing unit. Recently, systematic approaches have been developed to identify suitable and kinase-specific substrates to improve biosensor specificity, as exemplified by the use of position-scanning peptide library screening for AMPK substrates [14] and the kinase-interacting substrate screening (KISS) approach [15] for Rho-associated protein kinase (ROCK) substrates [16]. Work performed using various AMPK activity biosensors has since revealed distinct patterns of AMPK activity at subcellular locations induced by different input signals [14, 17], as well as the complex regulation of AMPK by PKA at the plasma membrane [18]. While biosensor optimization involves rational protein design and empirical, trial-and-error testing, additional systematic approaches have also been successfully employed to accelerate the creation of improved biosensors for pathways other than kinase signaling, including genetically encoded calcium indicators (GECIs) [19–21], genetically encoded voltage indicators (GEVIs) [22–24] and biosensors for neurotransmitters [25–27]. Most recently, for example, Villette *et al.* developed an efficient screening platform that combines an electroporation-based screening system and direct PCR (Polymerase Chain Reaction) transfection into mammalian cells [24]. Through multiple rounds of structure-guided combinatorial saturation mutagenesis, this work yielded the improved voltage sensor, ASAP3 (Figure 1C), with a higher response, favorable kinetics, and efficient membrane localization. In combination with ULoVE (ultrafast local volume excitation) two-photon microscopy, ASAP3 was able to report subthreshold membrane potentials with subcellular resolution in deep mouse brain regions during behavioral tasks.

Recent advances in the development and application of new and improved biosensors (Table 1) promise to not only illuminate signaling dynamics in time and space but also make a significant impact on the future of medicine, given the rapid growth of in vivo imaging using next-gen biosensors. Below, we highlight several exciting advances in which biosensors help address challenging biological questions and catalyze important scientific breakthroughs.

Refining localized biochemical activities: Super-resolution imaging with FLINC

Signaling molecules are spatially organized at multiple levels within cells, such as anchoring to organelle membranes, partitioning into membrane microdomains, associating with scaffold proteins, and assembling into protein complexes. A signaling “activity architecture” is thus built upon compartmentalization across length-scales ranging from a few nanometers

at the single-molecule level to hundreds of nanometers for large complexes [3]. Genetically encodable biosensors enable visualizing the dynamics of localized biochemical activities in live cells; however, the diffraction limit of visible light (200–250 nm) prevents the precise localization of biochemical activities on a comparable spatial scale via conventional light microscopy. On the other hand, super-resolution imaging methods based on patterned illumination or single-molecule localization have remarkably improved the spatial resolution beyond the diffraction limit. These methods, including stimulated emission depletion (STED), photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), stochastic optical fluctuation imaging (SOFI), and many others, have provided valuable biological insights into biomolecule localization with nanometer accuracy [28–32]; however, only a few examples address the challenges of visualizing biochemical activities in super-resolution, especially in the live-cell context. For example, live-cell imaging using STED microscopy requires particularly photostable fluorophores because of the high-energy illumination required for emission stimulation and depletion in STED mode. The intensity-based H_2O_2 biosensor, Hyper2, contains a highly photostable cpYFP that retains its sensitivity to H_2O_2. Using the brightness of Hyper2 as a readout for cellular H_2O_2 levels in STED imaging, Mishina *et al.* tracked H_2O_2 production in live cells and measured H_2O_2 microdomains as small as 100–200 nm [33, 34]. In addition, in a few cases of live-cell applications, **photoactivatible** [35], **photosconvertible** [36], and **photoswitchable** FPs [37] have been engineered to enable nanoscale imaging of protein-protein interactions based on **Bimolecular Fluorescence Complementation** (BiFC). However, BiFC is inherently irreversible and is limited to tracing *in situ* protein-protein interactions due to the relatively slow kinetics of FP reconstitution and chromophore maturation.

A general method to visualize dynamic biochemical activities, such as kinase activities, in super-resolution in living cells is highly desirable. To address this gap, Mo and coworkers introduced a new class of genetically encodable biosensors that enable visualization of activity dynamics and protein-protein interactions at a resolution of approximately 100 nm, up to threefold better than the resolution limit of conventional microscopy (200–250 nm) [38, 39]. These biosensors are based on the green FP Dronpa, a photoswitchable FP whose fluorescence can be reversibly switched on and off with 405 nm and 488 nm irradiation, respectively [40], and TagRFP-T, a non-switchable red FP [41] that exhibits weak, intrinsic fluorescence fluctuation (“blinking”) behavior, like many basic FPs [42]. Close proximity to Dronpa specifically enhances the rate and probability of TagRFP-T blinking, a phenomenon the authors termed **fluorescence fluctuation increase by contact** (FLINC) [38]. Dynamic changes in TagRFP-T blinking can be quantified via photochromic stochastic optical fluctuation imaging (pcSOFI), which utilizes statistical analyses of the fluorescence fluctuations in each pixel over time to generate super-resolution maps [43]. This imaging method, while achieving a spatial resolution below the diffraction limit, is user-friendly since data acquisition can be accomplished using any off-the-shelf microscope capable of capturing fluctuations. Because FLINC shows similar sensitivity to FP distance and orientation as FRET, the team was able to build a new series of FLINC-based biosensors for kinase activities, as well as protein-protein interactions, using FRET-based biosensors as a template. When combined with pcSOFI, these sensors allow changes in biochemical

activities, such as PKA and ERK activities, to be converted to “SOFI values”, which reflect the “intensity” of TagRFP-T blinking, and used to generate super-resolution activity maps.

Using the novel super-resolution PKA activity biosensor FLINC-AKAR (Figure 2A), Mo *et al.* were able to observe highly active puncta at the plasma membrane of living cells in response to cAMP production [38], suggesting that PKA activity is strictly confined to discrete microdomains (mean diameter of 350 nm) rather than being uniformly distributed across the plasma membrane (Figure 2B). These highly active PKA microdomains were found to colocalize with clusters of plasma membrane AKAP79/AKAP5, a predominantly membrane-localized scaffold that binds to RII PKA holoenzymes and other regulators of PKA signaling [38]. The authors further propose a working model where AKAP clustering selectively increases the local concentration of PKA regulatory subunits, leading to enhanced recapture of diffusive catalytic subunits and PKA activity compartmentalization. This refined model of compartmentalized PKA signaling suggests that the precise spatial regulation of other biochemical activities can also be dissected with this new class of FLINC biosensors.

FLINC biosensors are the first series of biosensors that report signaling activities with a resolution under the diffraction limit. The ability to precisely localize biochemical activities, combined with more refined structural information from localization-based super-resolution microscopy and multiplexed imaging at distinct subcellular localizations, provides the basis for unraveling the cell’s biochemical activity architecture. While being a highly compelling tool for elucidating signaling activities at the molecular scale, FLINC biosensors offer somewhat limited temporal resolution compared to conventional activity imaging. Specifically, each super-resolution activity map in a FLINC imaging time course is generated from a series of fluctuation images acquired under high-intensity illumination, which causes photobleaching. Periods of darkness, typically on the order of a few minutes, must therefore be included between each acquisition sequence in a time course to minimize this effect, which reduces the temporal resolution. However, it is hoped that engineering photostable FPs, improving imaging techniques, or new biosensor designs will help enhance the temporal resolution of super-resolution activity imaging.

It’s all about timing: New biosensors capture neurotransmitter release

A fundamental goal in neuroscience is to uncover the relationship between the neuronal signaling pathways that underlie brain function and behavioral outputs such as movement and learning. The billions of neurons in the brain communicate through trillions of **synapses**, where chemical signals called **neurotransmitters** are released to excite or inhibit specific target neurons, while various **neuromodulators** are secreted throughout the brain to modify neuronal activity. Understanding how neuronal firing and neurotransmitter release are coordinated in space and time to influence brain pathways and govern complex behavior remains a major challenge. A key step in addressing this challenge is to capture the in vivo dynamics of neurotransmitters with high spatiotemporal resolution, sensitivity and selectivity. Although widely used analytical chemistry techniques such as microdialysis and voltammetry permit the in vivo detection of neurotransmitters, they are limited by low temporal resolution on the order of minutes and low selectivity between neurotransmitters

with nearly identical redox potential, respectively, and both lack the ability to precisely target cells of interest [25, 26].

Great strides have been made in the recent development of genetically encodable FP-based biosensors for neurotransmitters. In contrast to the above analytical chemistry techniques, these biosensors offer high temporal precision and single-cell resolution. Many of these biosensors are developed using proteins [44–46] or receptors [25, 26, 47, 48] that are sensitive to neurotransmitter binding. For instance, Marvin *et al.* developed iGluSnFR, a single-FP sensor for glutamate (Glu), by inserting cpEGFP into the bacterial periplasmic glutamate/aspartate binding protein (GluBP). Upon Glu binding, GluBP is reconstituted, resulting in a ~5 fold fluorescence intensity increase [44]. iGluSnFR exhibits a high affinity and dynamic range to Glu, enabling visualization of Glu release at frequencies up to ~10 Hz (10 times per second) [44]. To resolve Glu dynamics at a higher frequency, Helassa *et al.* generated variants of iGluSnFR that have reduced Glu affinity by mutating residues in the GluBP binding pocket [45]. The resulting fast ($iGlu_f$) and ultrafast ($iGlu_u$) variants have comparable brightness to iGluSnFR, and their decreased Glu affinity enables more rapid glutamate dissociation from $iGlu_f$ and $iGlu_u$, allowing fast and sensitive imaging of synaptic Glu release events at 100 Hz [45].

Another strategy to engineer neurotransmitter biosensors is to use a GPCR (G-protein-coupled receptor) as the sensing unit, wherein the binding of neurotransmitters such as dopamine (DA) [25, 26, 47] or acetylcholine (Ach) [48] induces a conformational change in the GPCR. DA in particular plays an important role in motivation, movement, cognition, and reward-driven learning, and two series of DA biosensors have been developed to date: dLight [25] and GPCR-activation-based-DA ($GRAB_{DA}$) sensors [26]. By inserting cpEGFP into the third intracellular loop (IL3) of the human DA receptors D_1, D_2, and D_4, Patriarchi *et al.* generated and optimized variants of the dLight probe family (dLight1.1–1.5), which exhibit low-nanomolar to micromolar affinity for DA [25] (Figure 3A). Using a similar strategy, Sun *et al.* introduced cpEGFP into IL3 of the human D_2 DA receptor and systematically optimized the insertion site and linker sequence, while also tuning the affinity via mutation, yielding $GRAB_{DA1m}$ and $GRAB_{DA1h}$ with moderate (~130 nM) and high (~10 nM) binding affinity, respectively [26]. DA binding causes a highly specific and rapid increase in fluorescence intensity in both sensors, enabling spatiotemporally precise measurements of DA dynamics in live animals, and both sensors are also compatible with available optogenetic tools (Figure 3B).

The advent of these new DA biosensors has enabled detailed examinations of how DA supports brain function. In a recent study, Mohebi *et al.* shed light on how DA release from dopaminergic neurons (i.e., neurons that release DA) that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), one of four major dopaminergic pathways (e.g., the mesolimbic pathway, sometimes called the “reward” pathway), is regulated in the brains of rats performing a decision- making task [49]. In this pathway, the long axons of the VTA dopaminergic neurons extend to other parts of the brain, including the NAc, the dorsal striatum and the prefrontal cortex, where activation of these VTA neurons generates electrical signals (neuronal firing) that cause local DA release at these axon terminals. While DA neuron firing can encode reward prediction errors (RPEs) as a learning signal, DA

release can also reflect reward expectation as a motivation signal. To dissect how DA release is regulated to achieve these two distinct functions, e.g., learning and motivation, the authors measured DA cell firing in the VTA using single-cell optrode recording along with DA release in the NAc core using multiple approaches, including dLight, during the same task in freely moving rats. In this behavioral test, the authors observed elevated DA release only in the NAc and regions of the prefrontal cortex, but not in the dorsal striatum. Furthermore, DA release ramps up when the rate of reward increases, which motivates rats to participate in the task; however, neuronal activity in the VTA of rats was not affected, suggesting that motivation-coding DA release is independent of the firing activity of VTA DA neurons. By contrast, both VTA DA neuron spiking activity and DA release were linked to reward availability and the reward itself, suggesting that DA release associated with DA neuron firing encodes RPEs to promote learning. Because the dLight probe enables the detection of highly dynamic fluctuations in DA release on subsecond timescales, which correlates with both state values and RPEs, it suggests that DA release coupled with corresponding changes in the firing rates of DA neurons serves as a global “broadcast” to facilitate learning, whereas local DA release without DA neuron firing drives motivation (Figure 3C). Most recently, dLight has also been used to reveal reduced dopamine release in NF1 (neurofibromatosis type 1) mice, a model for an inherited neurological disorder [50], and to track fluctuations in DA release in the brains of mice across their sleep-wake cycle [51].

With their high dynamic ranges and optimized kinetics, the newly developed single FP-based biosensors for DA [25, 26], Glu [44, 45], Ach [48] and GABA [46] have enabled the quantification of neurotransmitter release dynamics in the brains of freely behaving animals, one of the most critical steps towards understanding the relationship between neuronal signaling pathways and brain functions. Improvements in biosensor performance (e.g., sensitivity and specificity), expanding the biosensor toolkit to simultaneously detect multiple signaling activities, and advanced microscopy techniques for in vivo functional brain imaging will further enable us to delineate how neuronal signaling patterns correlate with complex behavioral outputs.

Bioluminescence goes *in vivo*

Unlike fluorescence, **bioluminescence** involves light produced from a luciferase-catalyzed chemical reaction without the need for an external excitation source, thereby eliminating photobleaching, background interference, and autofluorescence. However, in vivo bioluminescence imaging has been hampered by low photon emission (i.e., quantum yield) [52], high tissue absorption and scattering in the visible spectrum [53], and low tissue permeability of luciferase substrates [54, 55]. Considerable efforts have therefore been devoted to improving bioluminescence imaging systems. For example, whereas firefly luciferase (FLuc) and Renilla luciferase (RLuc) were engineered as suitable tools for live-cell imaging, their signals are dim compared with fluorescence. Nano-lantern (Yellow NL) is a chimeric protein consisting of an enhanced RLuc with a bright FP, Venus [56]. Nano-lantern offers roughly 10 times the brightness of RLuc alone through BRET [57], where the excited-state energy produced during the RLuc-catalyzed oxidation of coelenterazine is nonradiatively transferred to the acceptor Venus, which emits photons with high efficiency, thus enhancing brightness [56]. By exploring different BRET acceptors, Takai *et al.* further

developed cyan and orange Nano-lantern variants (CNL and ONL) [58]. However, the RLuc substrate coelenterazine is less stable than the natural substrate, luciferin, and is rapidly consumed in cell culture media. Suzuki *et al.* developed 5 color variants of a brighter, enhanced Nano-lantern (eNL) [59] that incorporates the highly optimized NanoLuc variant of deep-sea shrimp *Oplophorus gracilirostris* luciferase with its optimal substrate, furimazine [60], which is more stable and produces lower autoluminescence than coelenterazine. Chu *et al.* also developed a cyan-excitable orange-red fluorescent protein, CyOFP1, to act as a BRET acceptor for NanoLuc, and the optimized fusion of CyOFP1 and NanoLuc, called Antares, improves BRET by approximately 3-fold and uses a lower concentration of substrate compared with FLuc [61]. Such improved sensitivity and expanded color variants allows multiplexed bioluminescence imaging [58, 59, 61] and tracking of the dynamics of subcellular structures [58, 59, 61], gene expression [58], and signaling events, such as intracellular calcium [58, 59, 62] and voltage change [63], and even extend the application to deep-tissue imaging in living mice [61, 64].

Perhaps the most striking example of efforts to extend the reach of bioluminescence is the recent development of the **AkaBLI system** for imaging in freely moving animals. Previously, Iwano and colleagues identified a synthetic analog of D-luciferin, AkaLumine [53] (Figure 4A), which emits near-infrared light when catalyzed by FLuc and has favorable biodistribution in deep organs and the ability to cross the blood-brain barrier (BBB). To develop a luciferase that favorably pairs with AkaLumine, the authors then performed directed evolution on the FLuc gene in the presence of AkaLumine to obtain Akaluc, an improved luciferase with enhanced catalytic efficiency, bright near-infrared emission (~52 times stronger than that from AkaLumine/FLuc), and high thermostability [65] (Figure 4B). The successful combination of Akaluc and AkaLumine enables the detection of bioluminescence signals in deep tissues (Figure 4C), for example, in lung tumor cells, as well as the tracking of a few hippocampal neurons in response to exposure to new environments. More impressively, the authors were able to monitor bioluminescence signals for more than one year without signs of toxicity in marmosets injected with an Akaluc-expressing AAV (adeno-associated virus) vector in the striatum [65]. The success of the AkaBLI system will certainly spark efforts to develop Akaluc-based biosensors to visualize signaling dynamics in unrestrained live animals.

A major bottleneck for the development of most bioluminescence imaging systems, however, is the requirement for exogenous luciferin, the luciferase-catalyzed oxidation of which triggers light emission. Engineering a bioluminescent system that produces both luciferase and luciferin could potentially solve this bottleneck. Two such bioluminescence systems have been identified to date: a bacterial bioluminescence system which utilizes the cell's abundant supply of reduced flavin mononucleotide as the luciferin [66, 67], and a fungal bioluminescence system that involves seven key genes for the biosynthesis of luciferase and luciferin [68, 69]. More recently, two groups transplanted the fungal bioluminescence pathway *in planta,* to achieve auto-luminescence and utilized this system to study gene expression *in planta* [70, 71]. Meanwhile, Srinivasan *et al.* took advantage of the bacterial bioluminescence system and developed Autonomous Molecular Biolumin Escent Reporter (AMBER), an indicator of membrane potential, for observing neural activity in

freely moving animals (*C. elegans*) with temporal resolution in seconds [72]. AMBER utilizes the membrane-bound *Ciona intestinalis* voltage-sensing domain (Ci-VSD) fused to an N-terminal flavin reductase phosphate (FRP) and a C-terminal BRET pair composed of a synthetic enhanced bacterial luciferase, eluxAB, and a bright yellow FP (YPet). As such, AMBER encodes genes to produce both luciferase and luciferin in situ and modulates luciferase activity as a function of membrane potential. An expanding toolbox of bioluminescence-based biosensors targeting signaling pathways continues to emerge [58, 59, 62, 63, 72]. Given the increased brightness of bioluminescence systems and lack of a requirement for external illumination, bioluminescence imaging will allow a range of in vivo applications to track signaling events in thick tissues and live animals, such as calcium and voltage dynamics, neurotransmitter release, and protein kinase activities.

Concluding remarks and future perspectives

As Sydney Brenner said, “Progress in science depends on new techniques, new discoveries and new ideas, probably in that order” [73]. Genetically encodable biosensors have become indispensable molecular tools that provide numerous insights into the dynamic regulation and function of signaling pathways that are entangled within complex signaling networks. Here, we have discussed recent advances in biosensor development, focusing on three recent examples of the development and application of fluorescent or bioluminescent biosensors. In these three examples, new biosensors or bioimaging systems were created to reveal crucial details of signaling dynamics and to address challenging biological questions or to achieve technological breakthroughs. These advances, combined with other exciting advances discussed elsewhere [74–77], will not only deepen our understanding of how signaling networks are coordinated to regulate the functions of living cells and organisms as part of the ongoing quest for scientific knowledge but will also open up avenues for exploring novel diagnostic, intervention, and treatment strategies for a myriad of diseases in the future. The field of genetically encodable biosensors is rapidly expanding now but is far from saturated with respect to target diversity, sensitivity and selectivity, spatiotemporal resolution, fluorescent and bioluminescent protein performance, imaging and processing techniques, and so on. For example, directed evolution has been widely used to increase biosensor dynamic range, multi-photon imaging technology has been developed and applied for tissue imaging [19, 24, 78, 79], and a proof-of-concept protease biosensor has also been recently developed for use with photoacoustic imaging to enable even deeper tissue penetration [80, 81]. Moving forward, future development of new biosensors may benefit from advances in computer-aided protein engineering [82, 83] to generate stable and bright FPs, tune binding affinities for analytes, and engineer larger dynamic changes. Future endeavors, including combining biosensor imaging with single cell-based sequencing, will contribute to further illuminate the intricate biochemical activity architecture with high spatiotemporal resolution both in cells and in vivo, and to unravel the complex etiology of human diseases.

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Box 1

The basics of biosensor design.

All genetically encodable biosensors are designed to convert a specific intracellular or extracellular signal, such as signaling enzyme activity, protein-protein interactions, and changes in second messenger concentrations, into a measurable fluorescent or bioluminescent readout. Therefore, biosensors enable the direct visualization of signaling molecule behavior within the native cellular context. Generally, biosensors consist of two essential components: a sensing unit and a reporting unit [84]. A sensing unit is responsible for detecting changes in the target biological events, while a reporting unit is responsible for converting these changes to measurable signals. In some cases, the functions of both the sensing unit and reporting unit can be fulfilled by a fluorescent protein (FP); for example, the binding of cellular analytes (such as chloride, iodide) [85] or intracellular pH changes can directly affect the chromophore of an FP and sensitize its fluorescence [86]. More commonly, the sensing unit is derived from endogenous proteins or peptide sequences that are sensitive to the target signaling event (e.g., phosphorylation or second messenger levels) [4], whereas the reporting unit consists of one or more FPs or luciferases configured to translate signaling-induced changes in the state of the sensing unit into one of several types of quantifiable signals, including Fluorescence Resonance Energy Transfer (**FRET**) [13, 87–90], fluorescence intensity [22–24, 27, 91, 92], excitation or emission ratio [5, 93, 94], and Bioluminescence Resonance Energy Transfer (**BRET**) [63, 95, 96].

The modular construction of biosensors has proven to be highly generalizable, as individual components can be optimized to enhance biosensor performance or specifically modified to monitor several major cellular signaling molecules, including protein kinases [90, 97], GTP hydrolases (GTPases) [98], and second messengers [4]. This modularity is exemplified by the design of kinase activity reporters, or KARs, in which a kinase substrate peptide paired with a phosphoamino acid binding domain (PAABD) is sandwiched between two FPs [77, 84]. Upon phosphorylation, the binding of the substrate peptide and PAABD generates a conformational change, leading to a change in FRET. Engineering different substrate-PAABD pairs has thus facilitated the development of a diverse family of FRET-based sensors for various protein kinases, including for PKA [13, 99–101], PKC [102], ERK [18, 101, 103], and CaMKII [104]. Many outstanding reviews [4, 77] are available to illustrate the design details of biosensors, and a comprehensive database for fluorescent biosensors can be found at biosensorDB.ucsd.edu.

Outstanding Questions

- How can we use computational algorithms, such as deep learning methods, to facilitate biosensor design and optimization?
- How can biosensor imaging approaches be integrated with powerful single-cell sequencing technology to provide a higher resolution view of cellular activity differences among individual cells within distinct microenvironments?
- In vivo imaging using biosensors represents one of the most exciting fields to study human diseases. How do we achieve precise quantification of biochemical signals in 4D dimensions from in vivo imaging?
- Multiplexed imaging promises the ability to simultaneously monitor the coordinated behaviors of multiple signaling molecules/pathways in time and space in a quantitative way. Is there a limit on the number of pathways that can be visualized with high sensitivity and specificity? How can we achieve a better understanding of signaling networks through multiplex imaging and computational modeling?
- Would signaling profiles obtained through imaging allow us to identify biomarkers for diagnosis and to track disease progression?

Highlights:

- Genetically encodable fluorescent and bioluminescent biosensors enable direct visualization of the spatiotemporal regulation of signaling networks within the native biological environment.
- The successful construction of a biosensor depends on the design of sensing and reporting units. Recent advances, such as new designs, new reporting units and screening approaches, have greatly accelerated the development of biosensors.
- Super-resolution FLINC biosensors break the diffraction limit and allow the construction of high resolution kinase activity maps.
- Newly developed biosensors help to capture the dynamics of neurotransmitter release.
- The successful pairing of an engineered luciferase with a synthetic substrate has led to the development of the AkaBLI system to enable highly sensitive cell tracking in vivo, as well as long-term bioluminescence imaging in the brains of freely moving animals.

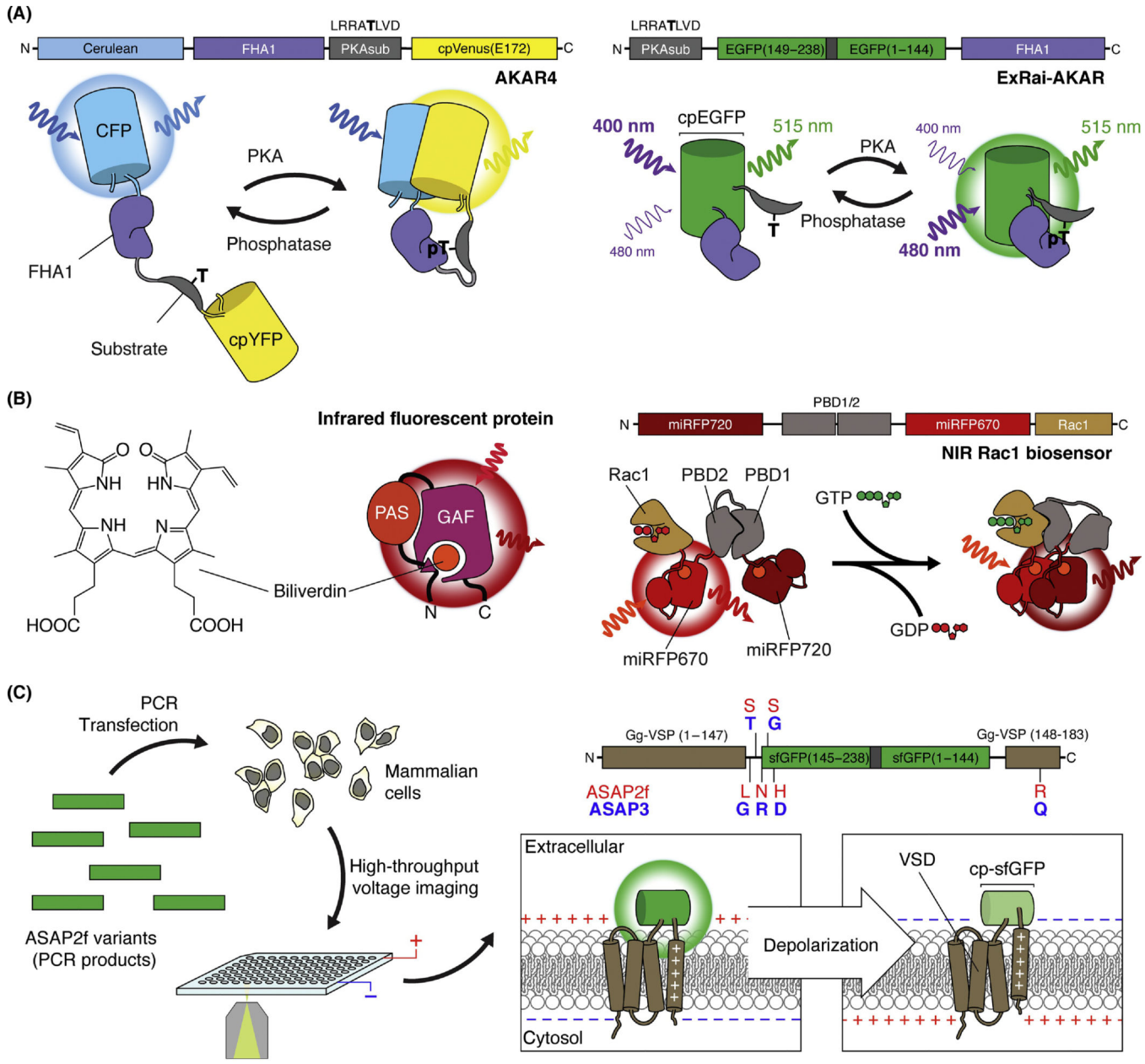

Figure 1. Advances in biosensor design and engineering.

(A) Left: FRET-based KARs such as A kinase activity reporter (AKAR) utilize an engineered molecular switch wherein the phosphorylation (e.g., T → pT) -dependent interaction between a substrate sequence for a kinase of interest (e.g., PKA) and a phosphoamino acid-binding domain (e.g., FHA1) modulates FRET between a pair of fluorescent proteins (FPs) such as Cerulean (e.g., CFP) and cpVenus (e.g., YFP). Right: Mehta *et al.* transplanted this molecular switch onto circularly permuted GFP (cpGFP), yielding a new suite of excitation ratiometric KARs (ExRai-KARs) [5]. As shown for ExRai-AKAR, PKA-mediated phosphorylation (e.g., T → pT) of the sensor induces a conformational change that shifts the maximum excitation wavelength from 400 nm to 480 nm. (B) Left: Several groups have utilized bacterial phytochromes (BphPs) to engineer

infrared fluorescent proteins (IFPs) capable of undergoing excitation and emission in the near-infrared (NIR) region of the electromagnetic spectrum. IFPs contain the PAS and GAF domains from BphP and utilize biliverdin, an endogenous heme catabolite, as their chromophore. Right: By engineering IFPs with different excitation/emission wavelengths, Shcherbakova *et al.* generated an all-NIR FRET pair, miRFP670/miRFP720, allowing them to construct NIR FRET sensors for monitoring Rac1 activation (shown), as well as PKA and JNK kinase activity [13]. (C) High-throughput approaches are highly desirable for accelerating biosensor engineering and optimization. Villette *et al.* recently developed a novel screening system (left) involving direct transfection of linear PCR products from a library of ASAP2f variants into mammalian cells, followed by high-throughput, automated electroporation-based voltage screening. Multiple rounds of screening ultimately yielded the enhanced voltage indicator ASAP3 (right), which contains 6 amino acid changes (highlighted in blue) with respect to ASAP2f (highlighted in red) [24]. ASAP-family sensors utilize the voltage-induced conformational change in the voltage-sensing domain (VSD) of chicken (*Gallus gallus)* voltage-sensitive phosphatase (GgVSP) to modulate the fluorescence intensity of circularly permuted superfolder GFP (cp-sfGFP), which is inserted between the 3rd and 4th helical segments of the VSD. Membrane depolarization results in a conformational change that decreases cp-sfGFP fluorescence intensity.

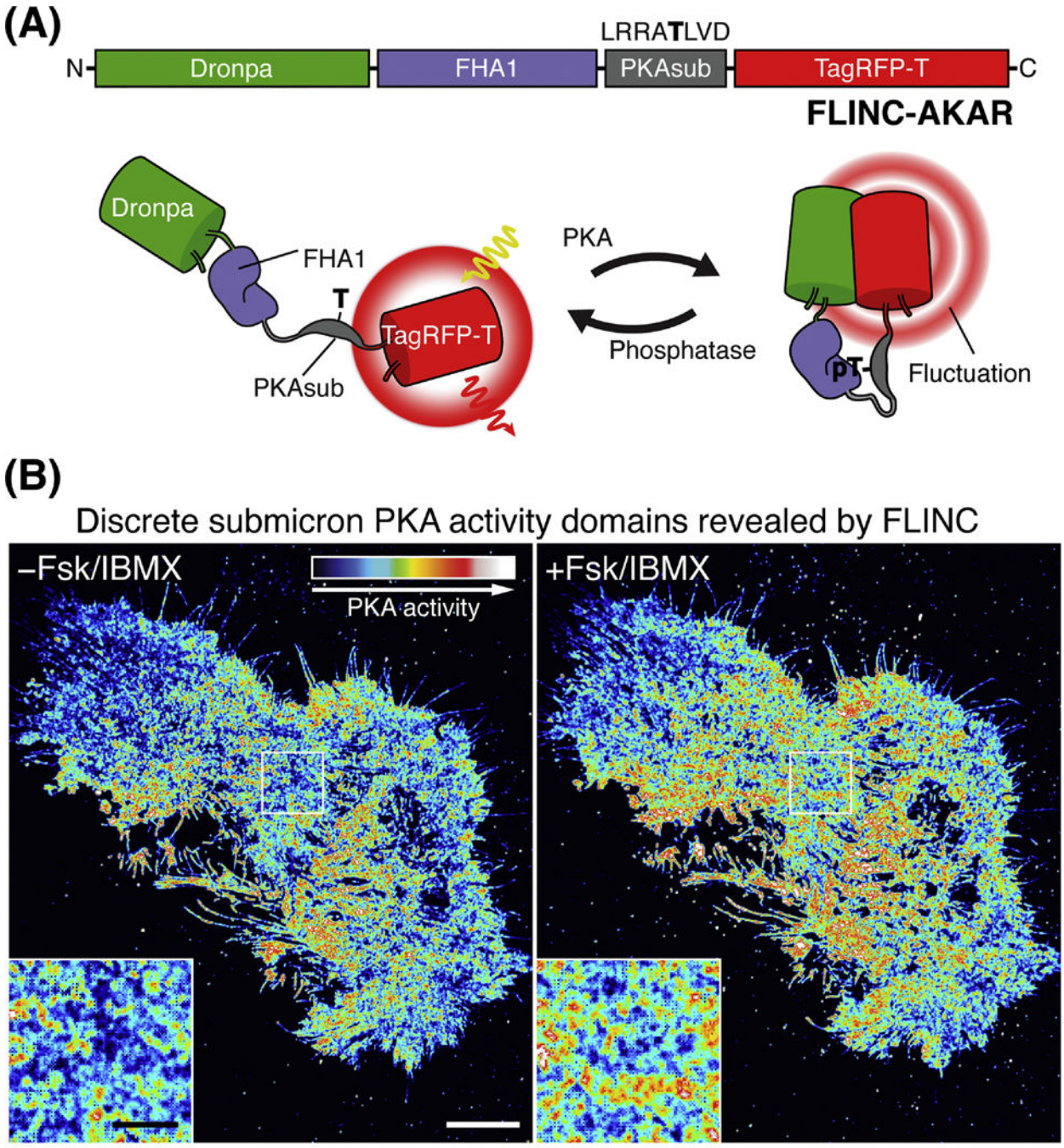

Figure 2. Super-resolution mapping of biochemical activities using FLINC.

(A) Mo *et al.* discovered a novel phenomenon, which they called fluorescence fluctuation induced by contact (FLINC), wherein the red fluorescent protein TagRFP-T exhibits a dramatic increase in spontaneous intensity fluctuations when in very close proximity to the green FP Dronpa [38]. Similar to FRET, FLINC can be utilized as a readout for genetically encoded biosensors through coupling to a conformational switch, thereby enabling super-resolution imaging of enzyme activities. For example, FLINC-AKAR contains a PKA substrate fused to FHA1, which are sandwiched between Dronpa and TagRFP-T. PKA-

dependent phosphorylation (T → pT) of the substrate induces binding by the FHA1 domain, leading to a conformational change and an increase in TagRFP-T intensity fluctuations. (B) Visualizing FLINC-AKAR in living cells using the super-resolution imaging technique pcSOFI yields a live-cell super-resolution map of PKA activity dynamics. In this example, HeLa cells expressing FLINC-AKAR localized to the plasma membrane were visualized before and after treatment with forskolin (Fsk) and 3-isobutyl-1-methyxanthine (IBMX) to activate PKA. Images generated through pcSOFI analysis reveal nanoscale domains of PKA activity (magnified in inset images) distributed non-uniformly across the cell surface. While PKA activity clearly increases after stimulation, as indicated by the pseudocoloring), the signal remains confined within these nanodomains, suggesting tight compartmentalization of PKA activity. Scale bars, 10 μm (full image) and 3 μm (inset)

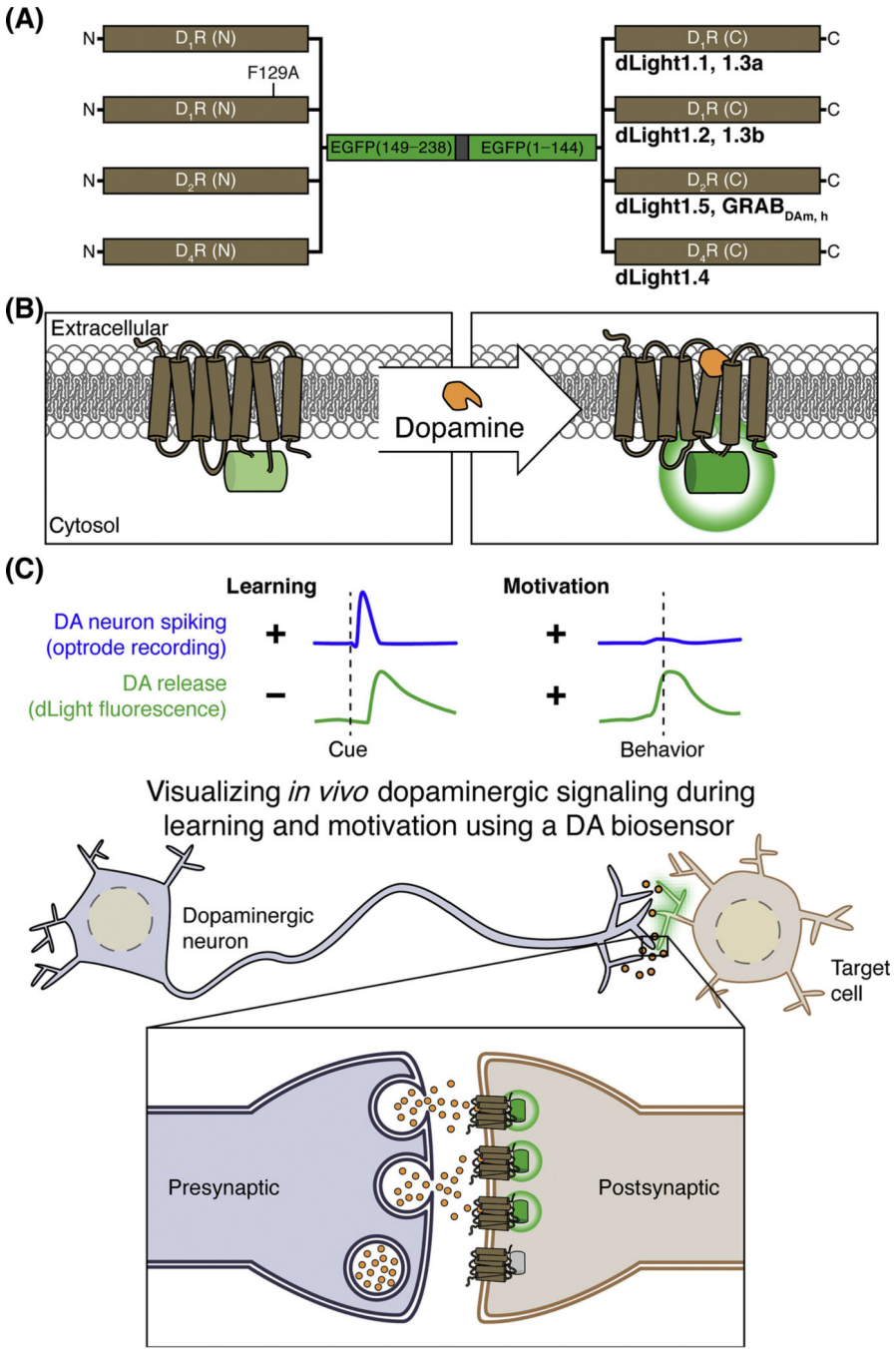

Figure 3. Novel sensors illuminate the dynamics of neurotransmitter signaling.

(A, B) Parallel work by Patriarchi *et al.* and Sun *et al.* has recently given rise to the dLight and $GRAB_{DA}$ families of biosensors for monitoring dopamine (DA) signaling. These sensors feature the insertion of cpEGFP within the 3rd intracellular loop of the D1, D2, or D4 type human DA receptors (A), such that the binding of DA to the receptor induces a conformational change that increases the fluorescence intensity of cpEGFP (B). (C) The advent of genetically encoded DA sensors is already helping to expand our understanding of DA neurotransmission in the brain. For example, Mohebi *et al.* recently used dLight as part

of their efforts to unravel the contributions of dopaminergic neuronal activity (e.g., spiking) and DA release in reward-driven learning and reward-seeking behavior (e.g., motivation) in rats [49]. Their work revealed that whereas both DA neuron firing (e.g., spiking, blue) and DA release (green) were associated with learning behavior, spiking-independent DA release was found to be associated with motivation.

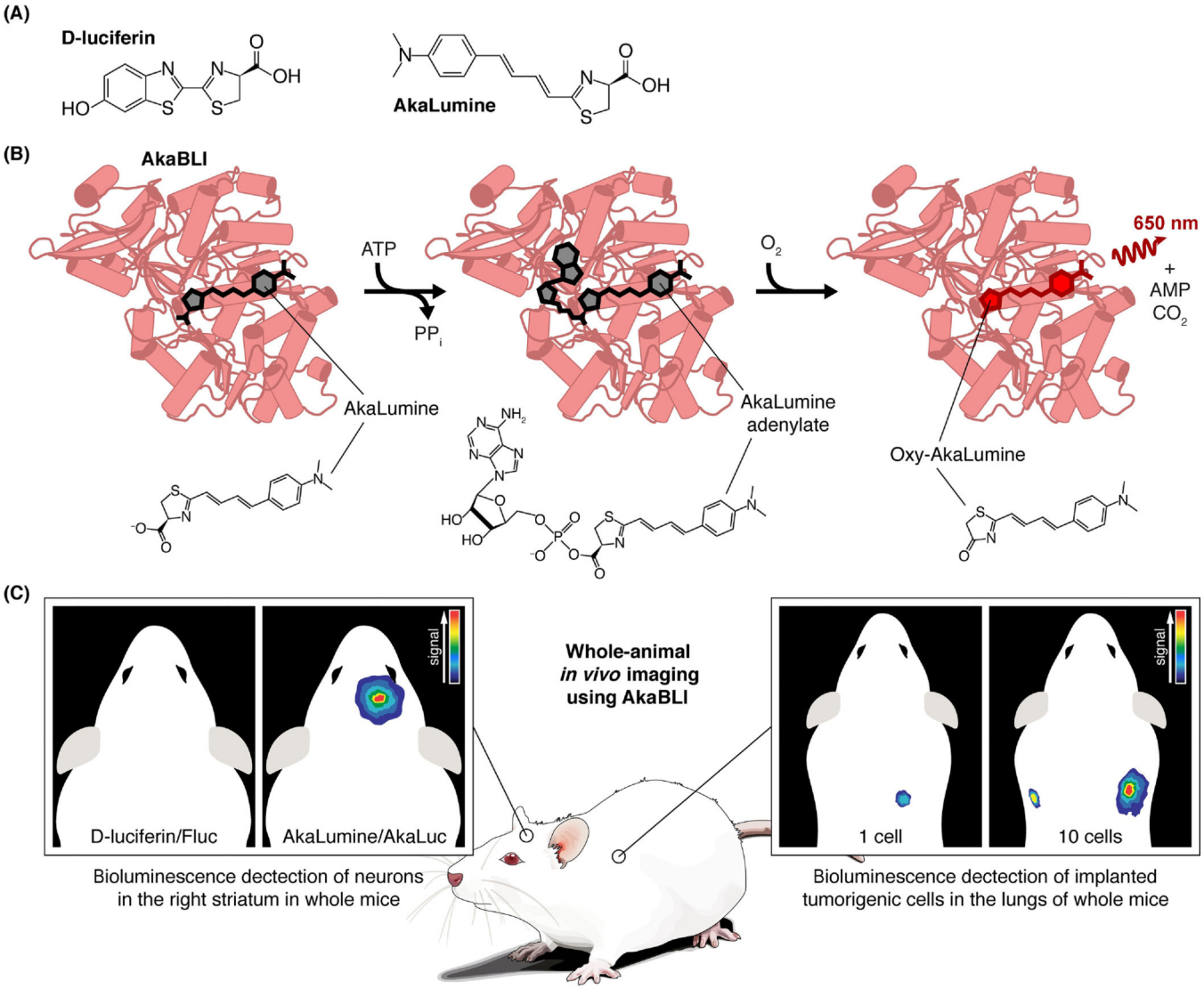

Figure 4. AkaBLI system for in vivo bioluminescence imaging.

(A) Chemical structures of D-luciferin and the red-shifted luciferase substrate AkaLumine. (B) The AkaBLI system consists of AkaLumine along with the novel luciferase Akaluc, which was engineered through directed evolution of FLuc to more efficiently catalyze the ATP-dependent oxidation of AkaLumine and increase light output. Illustration is modeled on the structure of FLuc (PDB 2D1R). Akaluc contains 28 amino acid substitutions with respect to FLuc. (C) Cell tracking in whole animals using AkaBLI.

Table 1

Recently developed fluorescent and bioluminescent biosensors with technical details, applications, advantages and limitations

Category	Feature	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
Fluorescent biosensors	Multiplexed imaging	ExRai-KARs (PKA, PKC, Akt)	Excitation ratiometric	cpGFP	widefield, multiplexed	Simultaneously tracking multiple signaling activities, including kinase activities, second messengers in live cells at distinct subcellular localizations.	• Ultrasensitive sensors • Compatible with multiplexed imaging • Good temporal resolution • Diffraction-limited spatial resolution	[5]
		RAB-AKARev, RAB-EKARev, RAB-ICUE	Intensity	ddFP[a]	widefield, multiplexed			[5, 105]
		blueKARs, sapphireKARs(PKA,PKC)	Intensity	cpBFP, cpSapphire	widefield, multiplexed			[5]
		iCasper	Intensity	cpIFP	in vivo	Tracking Caspase-3 activity, highlighting apoptotic cells during morphogenesis and tumor development in *Drosophila*.		[106]
	IFP-based biosensors	mKate2-DEVD-iRFP	ratiometric	iRFP	multiplexed	Detecting caspase-2 activation with EGFP-Bax translocation during apoptosis.	• Suitable for deep tissue and in vivo imaging because of low autofluorescence, light scattering and absorption • Compatible with multiplexed imaging • BphP derived IFPs are generally not bright, compared to GFP-like FPs. • Biliverdin is required for BphP derived IFP, limiting application in certain organisms and cell types.	[107]
		miSplit reporters NIR IkB reporter NIR Fucci	intensity/ BiFC	miRFP	widefield, SR-SIM[b], in vivo	Tracking protein-protein interactions, cell cycle progression, and NF-κB activation in live cells or animals		[108]
		Far-red and NIR cell cycle reporters	intensity/ BiFC	smURFP[c]	widefield, SR-SIM[b], in vivo	smURFP engineered to increase incorporation of biliverdin, brightness, and photostability		[109]
		NIR Rac1 biosensor, NIR JNKAR, NIR AKAR	NIR-FRET	miRFP670 miRFP720	multiplexed	Visualizing the antagonism between Rac1 and RhoA		[13]
	Newly engineered or enhanced biosensors via systematic approaches	AMPKAR	FRET	ECFP/ cpVenus Cerulean/ cpVen us	widefield	Tracking AMPK activity in response to different stimulation at distinct subcellular localizations in live cells.	• Systematic approaches as platforms to identify kinase-specific substrates, and to enhance biosensor performance.	[14, 17]
		ABKAR, Bim-ABKAR	FRET	Cerulean3/ cpVe nusCerulean/YP et	widefield			[18]
		AMPKAR-EV	FRET	SECFP/YPet	two-photon, in vivo	Delineate the regulation of AMPK in live cells and animals		[88]

Category	Feature	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
		Eevee-ROCK	FRET	ECFP/YPet	widefield	ROCK activation at early G1 phase and during apoptosis during cell cycle and apoptosis		[16]
		ASAP3	intensity	cpGFP	two-photon, ULoVE, in vivo	voltage dynamics, live animal imaging		[24]

Category		Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
Fluorescent biosensors	Super-resolution	FLINC-AKAR1, FLINC-EKAR1	FLINC	Photoswitchable FP, Dronpa and TagRFP-T	widefield, SOFI	Tracking activities of PKA and ERK at a structural resolution of about 100 nm	• Use reversibly switchable FPs • No need for specialized equipment • Structural resolution is below the limit of diffraction • Temporal resolution is higher than localization-based super-resolution imaging, e.g. PALM and STORM, but lower than conventional imaging • Requires specialized equipment • Requires highly photostable dyes or FPs • Balanced spatiotemporal resolution	[38]
		Venus-173-N-ORAI1 + STIM1-Venus-173-C	BiFC	Photoswitchable FP, cpDMVF, split at a.a. 181	widefield, refSOFI[d]	Visualizing interaction of receptor tyrosine kinase HER2&3, Dynamics of STIM1 and ORAI1 in ER-plasma membrane. 100 nm structural resolution in microscopy		[110]
		Hyper2	intensity	cpYFP	STED	Tracking the production of intracellular level of H2O2 at 100200 nm microdomains		[33]
		V1-ER + V2-Mito	BiFC	Venus, split	STED	Visualizing ER-Mitochondria association under stress		[111]
		Bak-rsEGFP2-N + Bcl-xL-rsEGFP2-C	BiFC	Photoswitchable FP, rsEGFP2, split at a.a.155	RESOLFT[e]	Visualizing heterodimerization interaction between Bcl- xL and Bak as well as the dynamics of the complex on mitochondria membrane in live cells		[112]
		RN-KRas G12D + CRaf-RBD-RC	BiFC	Photoactivatible FP, PA- mCherry1, split at a.a.159	PALM	Visualizing interactions of small GTPase Ras and its effector Raf		[113]
		mEosN-EF-Tu + MreB-mEosC	BiFC	Photoconvertible FP, mEos3.2, split at a.a.164	PALM	Visualizing localization dependent heterogeneous dynamics for MerB-EF-Tu.	• Requires highly photostable dyes or FPs • High spatial resolution • Limited temporal resolution • BiFC not suited for dynamic proteinprotein interaction	[36]
		EB1-PACF	BiFC	Photoactivatible FP, PA-GFP, split	PALM	Homodimerization of microtubule plus-end hub protein EB1		[114]
		mIN150-NFAT1+bJun-mIN(151–165)+bFos-mIC166; pa_s-mIN150 + pß1-	TFFC[f]	Photoactivatible FP, mIrisFP, split at a.a. 150, 166	PALM	Visualizing association of NFAT1, bJun, and bFos in Vero cells, as well as interaction of as, 01, and Y2 subunits of Gs ternary complex		[115]

Category		Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
		mIN(151–165) + and PY_2-mIC166						
		dLight 1.1–1.5	intensity			fast and sensitive detecting dopamine dynamics, compatible with in vivo imaging in freely behaving animals with in vivo imaging in freely behaving animals		[25]
		GRABDA1m, GRABDA1h; GRABDA2m, GRABDA2h	intensity	cpEGFP and DA receptor	in vivo, two-photon			[26, 47]
	Neuro-transmitter	iGluSnFR	intensity	cpEGFP and GluBP	in vivo, two-photon	imaging of synaptic glutamate release at up to 10-Hz	• High temporal precision and single cell resolution • Compatible with in vivo imaging • Suitable for multiplexed • Intensity-based biosensors offer large dynamic ranges and high SNR, but less quantitative than ratiometric biosensors	[44]
		$iGlu_f$, $iGlu_u$	intensity		two-photon	fast imaging of synaptic glutamate release events at 100-Hz		[45]
		GACh1.0, GACh2.0	intensity	cpEGFP and M_3R	widefield, one- and two-photon, in vivo	monitoring acetylcholine signals in vitro and in vivo.		[48]

Category	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
Bioluminescent probes	Nano-lantern (Ca^2+), Nano-lantern (cAMP), Nano-lantern(ATP1)	BRET	split luciferase RLuc8	wide-field	Tumor imaging in mice, tracking dynamics of calcium, cAMP, and ATP	• No need for external illumination • Eliminating photobleaching • Signals are highly specific to the monitored events due to low background • Compatible with multiplexed imaging • Dependent on cell permeability and biotransformation of luciferase substrates • Suitable for deep tissue and in vivo imaging in awake freely moving animals • Less invasive in vivo imaging than fluorescence imaging, but lower spatial resolution	[56]
	Mito-CNL(Ca^2+), ONL(Ca^2+)-H2B, CNL, YNL, ONL	BRET	split luciferase RLuc8	widefield, multiplexed	Visualizing subcellular structures, gene expression, and Ca^2+ dynamics at mitochondria and nucleus		[58]
	GeNL, CeNL, YeNL, ReNL, OeNL; GeNL(Ca^2+)	BRET	luciferase NLuc or split NLuc	widefield, multiplexed	Visualizing subcellular structures and Ca^2+ dynamics in cardiomyocytes		[59]
	Antares	BRET	CyOFP1 and NanoLuc	one- and two-photon, light-sheet, in vivo	Deep-tissue bioluminescence imaging of Antares with Ca^2+ indicator using both cell-based and live-animal models.		[77]
	Antares2	BRET	CyOFP1 and TeLuc	deep tissue	Deep-tissue bioluminescence imaging of an optimized Antares		[64]
	Orange CaMBI	BRET	Ca^2+ sensitive Antares	widefield, in vivo	Orange CaMBI reports calcium dynamics in		[62]

Category	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
					single cells and transgenic mouse		
	LOTUS-V	BRET	NanoLuc Venus	widefield	Imaging membrane voltage with optogenetic actuators and enabling long-term imaging		[63]
	AkaBLI	Bioluminescence	Akaluc and AkaLumine	bioluminescence lightsheet	Deep-tissue and in vivo bioluminescence imaging of tumor cells and neurons, and capable of long-term imaging		[65]
	AMBER	Bioluminescence	split luxAB	bioluminescence in vivo	Imaging membrane voltage in multiple worms moving in different directions	No need for exogenous substrate Lower temporal resolution than some of the other voltage sensors	[72]

Category	Feature	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
Fluorescent biosensors	Multiplexed imaging	ExRai-KARs (PKA, PKC, Akt)	Excitation ratiometric	cpGFP	widefield, multiplexed	Simultaneously tracking multiple signaling activities, including kinase activities, second messengers in live cells at distinct subcellular localizations.	• Ultrasensitive sensors • Compatible with multiplexed imaging • Good temporal resolution • Diffraction-limited spatial resolution	[20]
		RAB-AKARev, RAB-EKARev, RAB-ICUE	Intensity	ddFP[a]	widefield, multiplexed			[20, 105]
		blueKARs, sapphireKARs(PKA,PKC)	Intensity	cpBFP, cpSapphire	widefield, multiplexed			[20]
		iCasper	Intensity	cpIFP	in vivo	Tracking Caspase-3 activity, highlighting apoptotic cells during morphogenesis and tumor development in *Drosophila*.		[106]
		mKate2-DEVD-iRFP	ratiometric	iRFP	multiplexed	Detecting caspase-2 activation with EGFP-Bax translocation during apoptosis.	• Suitable for deep tissue and in vivo imaging because of low autofluorescence, light scattering and absorption • Compatible with multiplexed imaging • BphP derived IFPs are generally not bright, compared to GFP-like FPs. • Biliverdin is required for BphP derived IFP, limiting application in certain organisms and cell types.	[107]
	IFP-based biosensors	miSplit reporters NIR IkB reporter NIR Fucci	intensity/ BiFC	miRFP	widefield, SR-SIM[b], in vivo	Tracking protein-protein interactions, cell cycle progression, and NF-kB activation in live cells or animals		[108]
		Far-red and NIR cell cycle reporters	intensity/ BiFC	smURFP[c]	widefield, SR-SIM[b], in vivo	smURFP engineered to increase incorporation of biliverdin, brightness, and photostability		[109]
		NIR Rac1 biosensor, NIR JNKAR, NIR AKAR	NIR-FRET	miRFP670 miRFP720	multiplexed	Visualizing the antagonism between Rac1 and RhoA		[10]

Category	Feature	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
	Newly engineered or enhanced biosensors via systematic approaches	AMPKAR	FRET	ECFP/ cpVenus Cerulean/ cpVen us	widefield	Tracking AMPK activity in response to different stimulation at distinct subcellular localizations in live cells.		[42, 45]
		ABKAR, Bim-ABKAR	FRET	Cerulean3/ cpVe nusCerulean/Y P et	widefield			[32]
		AMPKAR-EV	FRET	SECFP/YPet	two-photon, in vivo	Delineate the regulation of AMPK in live cells and animals	• Systematic approaches as platforms to identify kinase-specific substrates, and to enhance biosensor performance.	[9]
		Eevee-ROCK	FRET	ECFP/YPet	widefield	ROCK activation at early G1 phase and during apoptosis during cell cycle and apoptosis		[44]
		ASAP3	intensity	cpGFP	two-photon, ULoVE, in vivo	voltage dynamics, live animal imaging		[18]

Category		Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
Fluorescent biosensors	Super-resolution	FLINC-AKAR1, FLINC-EKAR1	FLINC	Photoswitchable FP, Dronpa and TagRFP-T	widefield, SOFI	Tracking activities of PKA and ERK at a structural resolution of about 100 nm	• Use reversibly switchable FPs • No need for specialized equipment • Structural resolution is below the limit of diffraction • Temporal resolution is higher than localization-based super-resolution imaging, e.g. PALM and STORM, but lower than conventional imaging	[61]
		Venus-173-N-ORAI1 + STIM1-Venus-173-C	BiFC	Photoswitchable FP, cpDMVF, split at a.a. 181	widefield, refSOFI[d]	Visualizing interaction of receptor tyrosine kinase HER2&3, Dynamics of STIM1 and ORAI1 in ER-plasma membrane. 100 nm structural resolution in microscopy		[110]
		Hyper2	intensity	cpYFP	STED	Tracking the production of intracellular level of H2O2 at 100200 nm microdomains		[56]
		V1-ER + V2-Mito	BiFC	Venus, split	STED	Visualizing ER-Mitochondria association under stress	• Requires specialized equipment • Requires highly photostable dyes or FPs • Balanced spatiotemporal resolution	[111]
		Bak-rsEGFP2-N + Bcl-xL-rsEGFP2-C	BiFC	Photoswitchable FP, rsEGFP2, split at a.a.155	RESOLFT[e]	Visualizing heterodimerization interaction between Bcl- xL and Bak as well as the dynamics of the complex on mitochondria membrane in live cells		[112]
		RN-KRas G12D + CRaf-RBD-RC	BiFC	Photoactivatible FP, PA- mCherry1, split at a.a.159	PALM	Visualizing interactions of small GTPase Ras and its effector Raf	• Requires highly photostable dyes or FPs • High spatial resolution • Limited temporal resolution • BiFC not suited for dynamic proteinprotein interaction	[113]
		mEosN-EF-Tu + MreB-mEosC	BiFC	Photoconvertible FP, mEos3.2, split at a.a.164	PALM	Visualizing localization dependent heterogeneous dynamics for MerB-EF-Tu.		[59]

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		EB1-PACF	BiFC	Photoactivatible FP, PA-GFP, split	PALM	Homodimerization of microtubule plus-end hub protein EB1		[114]
		mIN150-NFAT1+bJun-mIN(151–165)+bFos-mIC166; pa_s-mIN150 + pß1-mIN(151–165) + and PY_2-mIC166	TFFC[f]	Photoactivatible FP, mIrisFP, split at a.a. 150, 166	PALM	Visualizing association of NFAT1, bJun, and bFos in Vero cells, as well as interaction of as, 01, and Y2 subunits of Gs ternary complex		[115]
	Neuro-transmitter imaging	dLight 1.1–1.5	intensity					[49]
		GRABDA1m, GRABDA1h; GRABDA2m, GRABDA2h	intensity	cpEGFP and DA receptor	in vivo, two-photon	fast and sensitive detecting dopamine dynamics, compatible with in vivo imaging in freely behaving animals		[50, 70]
		iGluSnFR	intensity	cpEGFP and GluBP	in vivo, two-photon	imaging of synaptic glutamate release at up to 10-Hz	• High temporal precision and single cell resolution • Compatible with in vivo imaging • Suitable for multiplexed • Intensity-based biosensors offer large dynamic ranges and high SNR, but less quantitative than ratiometric biosensors	[67]
		iGluf, iGluu	intensity		two-photon	fast imaging of synaptic glutamate release events at 100-Hz		[68]
		GACh1.0, GACh2.0	intensity	cpEGFP and M_3R	widefield, one- and two-photon, in vivo	monitoring acetylcholine signals in vitro and in vivo.		[71]

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	Mito-CNL(Ca^2+), ONL(Ca^2+)-H2B, CNL, YNL, ONL	BRET	split luciferase RLuc8	widefield, multiplexed	Visualizing subcellular structures, gene expression, and Ca^2+ dynamics at mitochondria and nucleus	• No need for external illumination • Eliminating photobleaching • Signals are highly specific to the monitored events due to low background • Compatible with multiplexed imaging • Dependent on cell permeability and biotransformation of luciferase substrates • Suitable for deep tissue and in vivo imaging in awake freely moving animals • Less invasive in vivo imaging than fluorescence imaging, but lower spatial resolution	[81]
	GeNL, CeNL, YeNL, ReNL, OeNL; GeNL(Ca^2+)	BRET	luciferase NLuc or split NLuc	widefield, multiplexed	Visualizing subcellular structures and Ca^2+ dynamics in cardiomyocytes		[82]
	Antares	BRET	CyOFP1 and NanoLuc	one- and two-photon, light-sheet, in vivo	Deep-tissue bioluminescence imaging of Antares with Ca^2+		[77]

Category	Name of Biosensor	Readout	Biosensor Engineering	Imaging Techniques	Application	Advantages and limitations	Ref.
					indicator using both cell-based and live-animal models.		
	Antares2	BRET	CyOFP1 and TeLuc	deep tissue	Deep-tissue bioluminescence imaging of an optimized Antares		[86]
	Orange CaMBI	BRET	Ca^{2}+ sensitive Antares	widefield, in vivo	Orange CaMBI reports calcium dynamics in single cells and transgenic mouse		[85]
	LOTUS-V	BRET	NanoLuc Venus	widefield	Imaging membrane voltage with optogenetic actuators and enabling long-term imaging		[22]
	AkaBLI	Bioluminescence	Akaluc and AkaLumine	bioluminescence lightsheet	Deep-tissue and in vivo bioluminescence imaging of tumor cells and neurons, and capable of long-term imaging		[87]
	AMBER	Bioluminescence	split luxAB	bioluminescence in vivo	Imaging membrane voltage in multiple worms moving in different directions	No need for exogenous substrate Lower temporal resolution than some of the other voltage sensors	[94]

[a] ddFP: dimerization-dependent FP.

[b] SR-SIM: super-resolution structured illumination microscopy.

[c] smURFP: small ultra red FP.

[d] refSOFI: reconstituted fluorescence-based SOFI.

[f] TFFC: Three-Fragment Fluorescence Complementation, is an extension of the BiFC technology.

[e] RESOLFT: REversible Saturable OpticaL Fluorescence Transitions, optical super-resolution fluorescence microscopy techniques.